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TITLE: An Oral DNA vaccine Encoding Endoglin Eradicates Breast
        Tumors by Blocking Their Blood Supply

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Among novel strategies preventing or slowing breast cancer metastasis are those blocking continuous new blood vessel growth, i.e. angiogenesis in the tumor vasculature mediated by proliferating endothelial cells lining such vessels. These cells overexpress a glycoprotein called endoglin which stimulates such vessels. We successfully constructed and evaluated an oral endoglin-based DNA vaccine and demonstrated its capability to induce a robust $CD8^+$ T cell response that specifically killed proliferating endothelial cells in the breast tumor vasculature. This, in turn, was shown to markedly decrease tumor angiogenesis resulting in a decisive suppression of breast tumor metastases leading to doubling in life-span of successfully vaccinated mice in a prophylactic breast tumor metastasis model.

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INTRODUCTION:

There is a real need to develop novel and effective treatments for metastatic breast cancer since this disease leads to more than 40,000 breast cancer related deaths per year among American women. It is well established from locoregional treatment of breast cancer that many patients with localized disease suffer from micrometastases. Surgical methods or radiation do not destroy these microscopic tumors which may grow to relevant size, causing multi-organ dysfunction and death. Among novel therapies preventing or slowing breast cancer metastasis are those blocking continuous blood vessel growth, i.e. angiogenesis, in the tumor vasculature mediated by proliferating endothelial cells lining such vessels. These cells overexpress a glycoprotein called endoglin which stimulates blood flow to tumors. Once endoglin is prevented from binding transforming growth factor (TGF-beta) on proliferating endothelial cells, and once such cells are selectively killed, effective blood flow to tumors is halted, resulting in their starvation, followed by growth inhibition and death. One of the more effective means of killing these proliferating endothelial cells in the tumor vasculature is to suppress tumor angiogenesis via the induction and proliferation of potent cytotoxic, T lymphocytes, specifically directed against endoglin. Significantly, such T-killer cells are capable of a memory response against their target, and if needed, can kill cells expressing it over prolonged time periods, thus preventing the recurrence of cancer. This killing process is quite intricate and requires small, effectively processed fragments of endoglin to be complexed with major histocompatability antigens to be then presented by activated dendritic cells to naïve T cells.

Among the strategies to be used to optimize T cell-mediated killing of proliferating endothelial cells in the breast tumor vasculature is to construct a DNA vaccine encoding the entire endoglin gene and to determine its protein expression and anti-angiogenic efficacy/anti-tumor activity against breast cancer. This is done by specific oral delivery of this vaccine via double attenuated, non-infectious *Salmonella typhimurium* to secondary lymphoid tissues, i.e. Peyer's patches in the small intestine. This, in turn, assures markedly enhanced immunogenicity and an induction of robust cytotoxic T cell responses against endoglin. The development and standardization of orthotopic breast tumor models in syngeneic mice together with the establishment of spontaneous and experimental lung metastasis models of breast cancer facilitated the initial evaluation of our vaccine's anti-tumor efficacy and its ability to block tumor angiogenesis.

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<u>BODY:</u>

Our goals in the first fiscal year were to: a) develop and standardize orthotopic breast tumor models together with the establishment of spontaneous and experimental lung metastases models; b) construct a DNA vaccine encoding endoglin and determine its protein expression by Western blotting and anti-angiogenic efficacy in Matrigel assays; c) evaluate the *in vivo* anti-angiogenic/anti-tumor activity of this DNA vaccine in D2F2 fat pad orthotopic tumors in spontaneous as well as experimental mouse tumor models with emphasis on suppression of breast tumor metastases.

Generally speaking, our outline in the SOW remained very much on schedule. Initially, we determined endoglin expression on murine breast tumor cell lines (D2F2, 4T1), endothelial cells (HEVc) and normal tissue (spleen, lung). RT-PCR results indicated that, as expected, HEVc endothelial cells expressed endoglin while D2F2 tumor cells lacked expression. The 4T1 tumor cell is a noteworthy exception, since unlike the vast majority of tumor cell lines, it expresses endoglin (CD105) by RT-PCR and FACS analysis (Fig. 1 A, B). Immunostaining revealed that CD105 is well expressed in lungs of D2F2 tumor bearing mice, particularly in proliferating endothelial cells but is not expressed by normal lung tissues (Fig. 1C). The pCMV/myc/cyto expression vector encoding endoglin was successfully constructed as shown schematically in Fig. 2A and correct protein expression of endoglin was demonstrated by Western blotting (Fig. 2B). FACS analysis indicated that endoglin in COS-7 cells, transfected with the expression vector is expressed as protein on the cell surface, similar as on HEVc endothelial cells (Fig. 2C).

We could also demonstrate that our vaccine delivery system, using double attenuated *Salmonella typhimurium* (dam-;AroA-), was successful in delivering the plasmid to mouse Peyer's patches *in vivo* and consequently to prime specific T cells which can recognize epitopes expressed on CD11$^+$ dendritic cell subpopulations which did express endoglin intracellularly in Peyer's patches 24 h after oral delivery of attenuated *Salmonella typhimurium* carrying pCMV-endoglin (Fig. 3)

Endoglin vaccination was shown to effectively induce *in vivo* suppression of D2F2 breast tumor metastases. In fact, in a prophylactic setting significant suppression of lung metastases was evident (Fig. 4A) and life-span of successfully vaccinated mice essentially doubled when compared to controls (Fig. 4B).

A series of *in vivo* depletions of CD4$^+$ and CD8$^+$ T cells clearly demonstrated that depletion of CD4$^+$ T cells had no effect, whereas depletion of CD8$^+$ T cells completely abrogated the anti-tumor effect of pCMV-endoglin (Fig. 5), indicating that the CD8$^+$ T cell population is responsible for the major anti-tumor effects induced by the vaccine in the prophylactic mouse tumor model. We could also demonstrate that endoglin-expressing endothelial cells were specifically eliminated by CTL induced by the oral endoglin-based DNA vaccine as indicated by both ELISPOT and CTL assays. Thus, ELISPOT assays indicated an increased frequency of specific, activated T cells that can recognize epitopes derived from endoglin$^+$ endothelial cells (HEVc) (Fig. 6A). The endothelial cells were specifically susceptible to T cells obtained from

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vaccinated mice, in contrast to such cells obtained from control mice which were ineffective (Figs. 6 B&C).

Finally, we could demonstrate that our endoglin-based DNA vaccine is capable of markedly suppressing angiogenesis *in vivo* in a Matrigel assay using fluorescent *Bandeiraea simplicifolia* lectin as an indicator. We could also quantify the extent of angiogenesis expression by measuring fluorescence at 490nm of extracts from 100µl matrigel plugs removed from immunized and control mice (Fig. 7). The evidence obtained thus far indicates that our oral endoglin-based DNA vaccine induces a CD8$^+$ T cell response capable of killing proliferating endothelial cells in the D2F2 breast tumor vasculature, resulting in inhibition of tumor angiogenesis and leading to marked suppression of pulmonary metastases of breast cancer in a prophylactic setting. Task #1 (months 1 – 15) was thus essentially completed.

KEY RESEARCH ACCOMPLISHMENTS:

1) Murine endoglin expression was demonstrated on endothelial cells but not on D2F2 breast carcinoma cells or on normal spleen and lung tissues. The 4T1 breast carcinoma cell line proved to be an exception to the rule as it expressed endoglin.

2) An expression vector encoding the entire endoglin gene was constructed and shown to correctly express endoglin protein in transfected C0S-7 cells, including surface expression of this molecule.

3) The oral endoglin-based DNA vaccine carried by attenuated *Salmonella typhimurium* specifically delivered the plasmid to mouse Peyer's patches where cryosections obtained 24 h after vaccination revealed intracellular endoglin expression by CD11$^+$ dendritic cells.

4) The endoglin-based DNA vaccine induced marked suppression of pulmonary metastases of breast carcinoma and doubling in life-span of successfully vaccinated mice.

5) *In vivo* depletion experiments of CD4$^+$ and CD8$^+$ T cells indicated that CD8$^+$ T cells were responsible for the anti-tumor effect induced by the endoglin-based DNA vaccine.

6) ELISPOT and CTL assays demonstrated that endoglin-expressing endothelial cells could be eliminated by CTLs induced by the oral endoglin-based DNA vaccine.

7) The endoglin-based DNA vaccine induced a robust CTL response which killed proliferating endothelial cells in the breast tumor vasculature which markedly suppressed tumor angiogenesis.

REPORTABLE OUTCOMES:

The reportable outcome and results from the first fiscal year of this grant are as follows:

1) A manuscript draft entitled "Targeting of endoglin with a DNA-based vaccine induces T cell-mediated suppression of angiogenesis and breast tumor metastases' will soon be submitted for publication.

2) An invited talk was given in a minisymposium at the American Association of Cancer Research meeting in Anaheim, CA. April 16-20, 2005.

3) A poster presentation of this work will be given at the DOD-sponsored Era of Hope meeting at Philadelphia, PA., June 8-9, 2005.

4) Based on our encouraging preliminary data with this endoglin-based DNA vaccine, we plan further experiments to strengthen our results for publication and to obtain further data in the spontaneous metastasis model that should lead to additional publications. Emphasis will be on prolonging T cell memory and on effective inhibition of breast tumor recurrence.

CONCLUSIONS:

Our objectives in the first fiscal year of this grant were met as we constructed and critically evaluated an oral endoglin-based DNA vaccine and demonstrated initially its capability in evoking a robust T cell-mediated immune response capable to overcome peripheral T cell tolerance against the endoglin self-antigen.

Also, we were able to demonstrate at least in a prophylactic setting in syngeneic BALB/c mice that our DNA vaccine suppressed experimental pulmonary metastases of D2F2 breast carcinoma. We could also show that this was achieved by inducing a robust CD8$^+$ T cell response against endoglin which resulted in the killing of proliferating endothelial cells in the breast tumor vasculature. This, in turn, lead to inhibition of tumor angiogenesis resulting in marked suppression of metastic breast tumor growth.
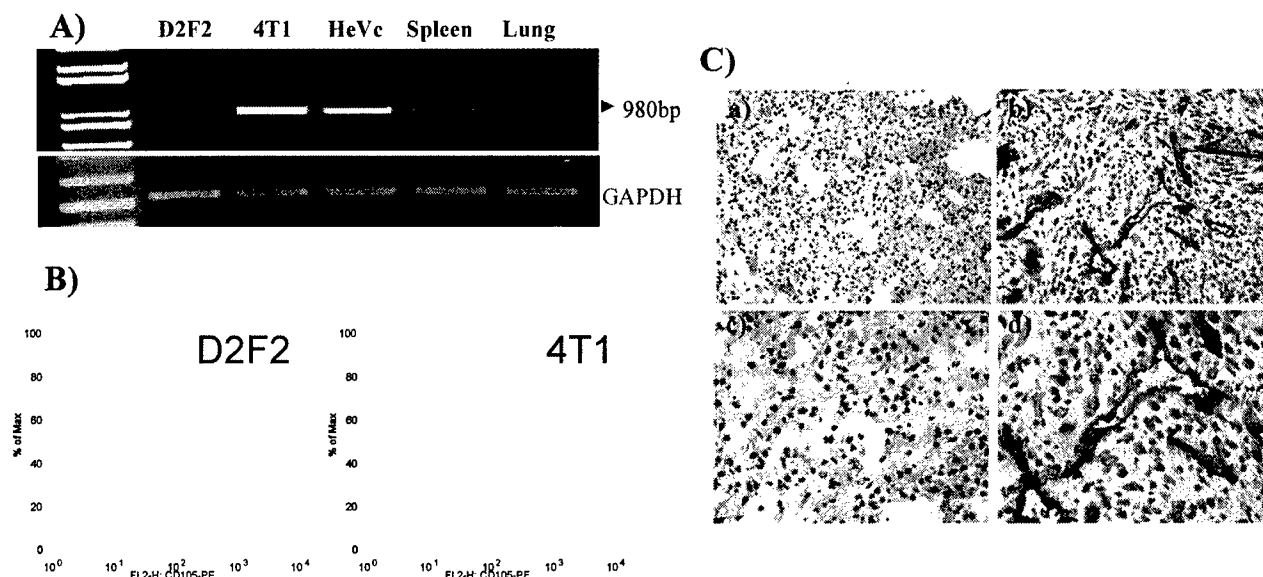
APPENDICES:

A)

	D2F2	4T1	HeVc	Spleen	Lung

▶ 980bp

GAPDH

B)

D2F2

4T1

% of Max

% of Max

FL2-H: CD105-PE

FL2-H: CD105-PE

C)

a) b)

c) d)

Fig. 1. Examination of CD105 expression on cells. Comparison of expression levels on normal lungs **(a, c)** and lungs of tumor-bearing mice **(b, d)**. **(A)** RT-PCR was performed to establish the expression of CD105 at the RNA level and **(B)** CD105 protein expression on the cell surface was examined by FACS analysis. **(C)** Immuno-staining was performed to compare expression levels of CD105 in normal lungs **(a, c)** with that of CD105 in lungs of tumor bearing mice **(b, d)**. Upper panels **(a, b)** low magnitude (X20) and panels **(c, d)** high magnitude (X40).

A)

Vector construction

NcoI Murine Endoglin gene XhoI

pCMV/myc/cyto
4.9 kb

B)

CMV-mEndo CMV Cos7 (KDa)

− 181.8
− 115.5
− 82.2

Actin

C)

CMV
0.71   3.84

25.6   69.9
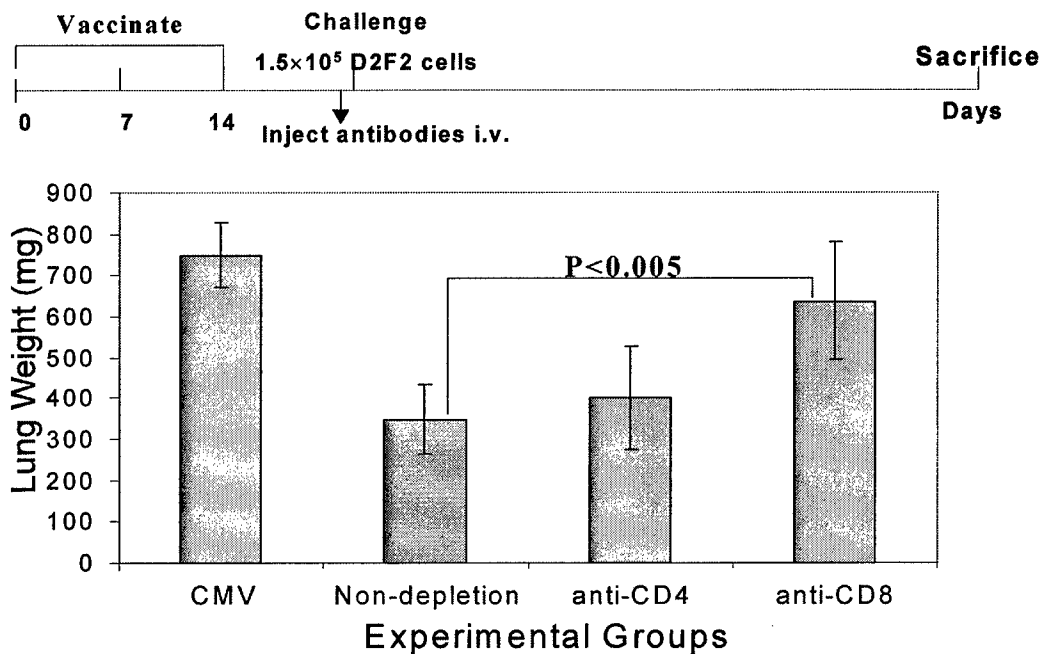
CMV-endoglin
3.86   29.1

19.6   47.5
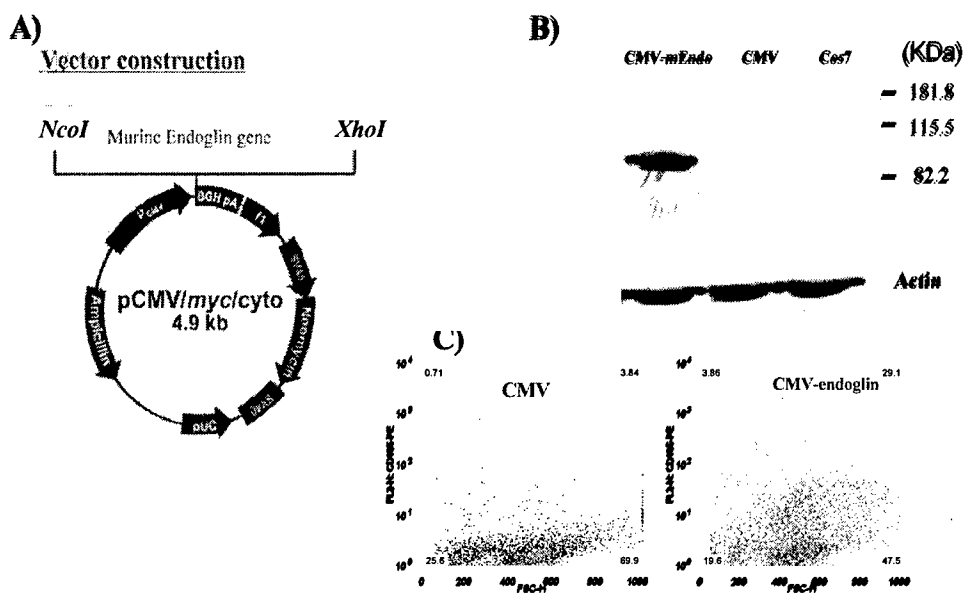
Fig. 2. Schematic of a vector encoding endoglin (A) and protein expression confirmed by Western blot and FACS analyses. (B) Western blots were performed to test endoglin protein expression in cell lysates from COS-7 cells transfected with either pCMV-endoglin or pCMV vector. (C) Endoglin expression on the cell surface was also examined by FACS analyses of COS-7 cells transfected with either pCMV-mEndo or pCMV vector as a negative control.

CD11c	Endoglin	CD11c+Endoglin	
			CMV-Endoglin
			CMV
			(X60 magnitude)
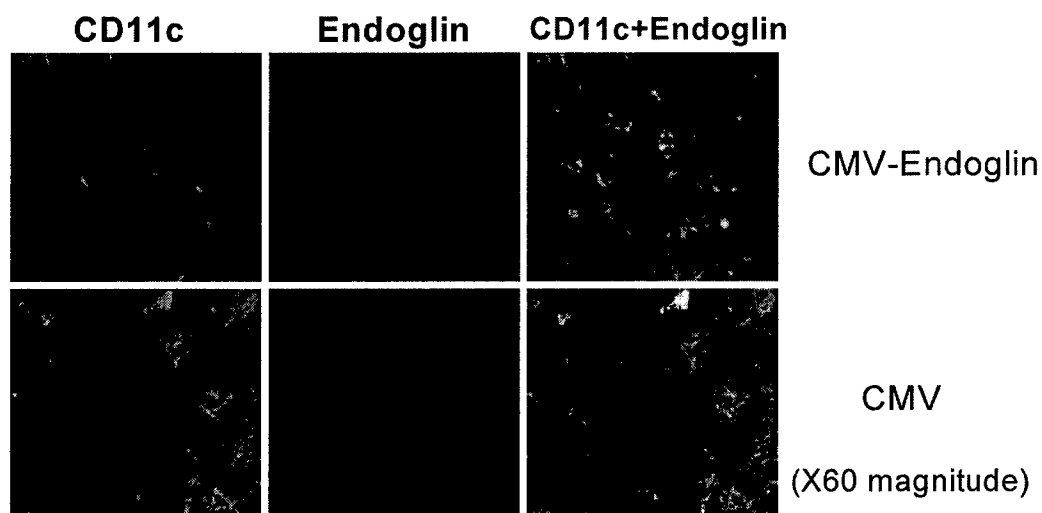
Fig. 3. Confocal microscopic analysis of endoglin expression of CD11c$^+$ cells in Peyer's patch (PP). PP cryosections were prepared from mice 24 h after delivery of attenuated Salmonella. Sections were stained with antibodies specific for CD11c (green) and endoglin expression (red), and examined by confocal microscopy.
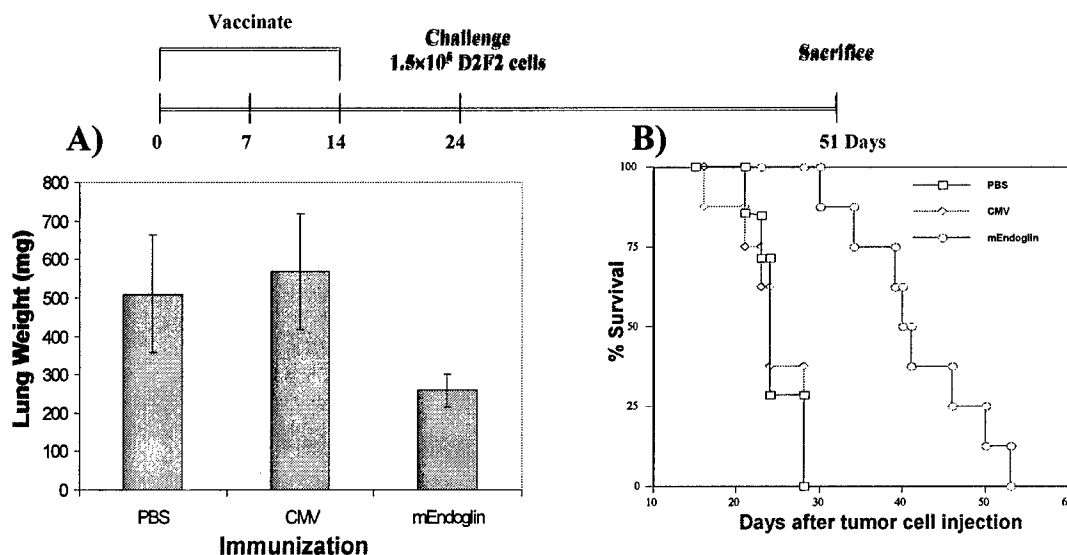
Fig. 4. Suppression of pulmonary metastases of D2F2 breast carcinoma by the oral DNA encoding endoglin vaccine in prophylactic models. (A) Lung metastases were induced in the prophylactic setting by i.v. injection of 1.5×10^5 D2F2 cells 1 week after the last of three immunizations administered by gavage at one week intervals. Experiments were terminated 27 days after tumor cell inoculation and the extent of pulmonary tumor metastases evaluated. (B) Survival days of mice vaccinated with endoglin DNA was determined.

Fig. 5. Abrogation of D2F2 metastasis suppression induced by an oral endoglin-based vaccine in mice depleted of CD8$^+$ T cells. Mice were immunized 3 times at weekly intervals with pCMV-endoglin. Monoclonal antibodies, anti-CD8 or anti-CD4, were injected i.v. 10 days after the last immunization. The next day, mice were challenged with D2F2 breast tumor ($1.5{\times}10^5$ cells/mouse) and antibodies injected i.p. once a week. Mice were sacrificed 25 days after tumor cells challenges and their lungs weighted to determine any suppression of pulmonary metastases.
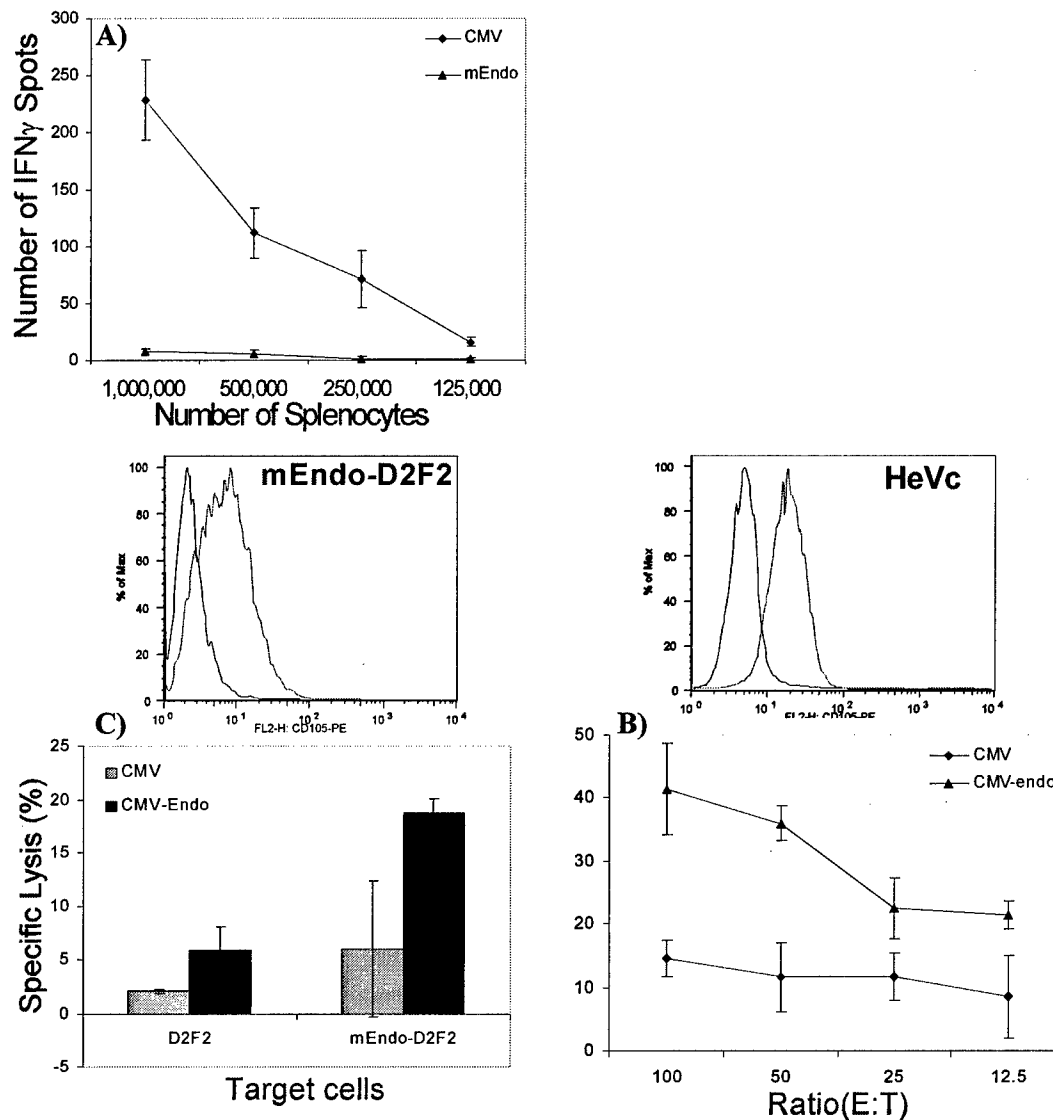
Fig. 6. *In vitro* CTL and ELISPOT assays. **(A)** ELISPOT assays were performed with an ELISPOT kit (BD Biosciences, Pharmingen) according to the instructions provided by the manufacturer. (Middle panels) FACS analyses to examine endoglin expression of two target cells. **(B)** Susceptibility of endothelial target cells, HeVc, to CTL killing at various E/T ratios. **(C)** CTL assays for testing of target cells either endoglin positive or negative. Data indicate representative CTL lysis mediated at E:T ratio of 1:25.
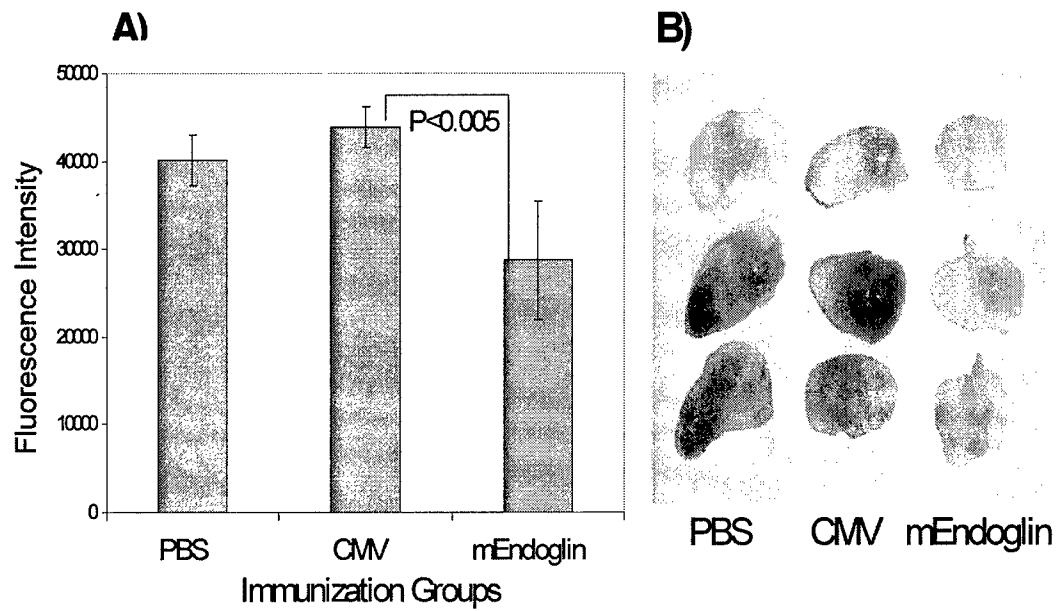
Fig. 7. Suppression of angiogenesis by the endoglin-based DNA vaccine. (A) Average fluorescence of 100μg Matrigel plugs from each experimental group of mice is depicted by the bar graphs (n=4). (B) Anti-angiogenesis as evident in Matrigel plugs of 100μl obtained from mice vaccinated either with pCMV-endoglin, pCMV or PBS .